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ANTIOXIDATIVE RESPONSES OF MICROALGAE TO HEAVY METALS

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Abstract

Microalgae are unicellular free living entities and therefore their responses to excess of heavy metals must be faster and more efficient than those in vascular plants protected by various types of tissues. Up to date, numerous studies reported metal bioaccumulation potential of algae but metabolic responses have relatively rarely been monitored. Here I provide basic overview of quantitative changes of ascorbic acid (AA), reduced glutathione (GSH), phytochelatins (PCs) and selected related enzymes (ascorbate peroxidase and glutathione reductase) in some common microalgae exposed to various metals (cadmium mainly). Despite various culture and exposure conditions, some common signs of metal toxicity (including e.g. enhancement of phytochelatin biosynthesis) are clearly identifiable in algae. Other metal chelators such as organic acids are also briefly mentioned. Comparison with macroalgae, mosses and vascular plants is discussed in terms of basal values and evolutionary similarities.

Key words: antioxidants, heavy metals, oxidative stress

Abbreviations: APX – ascorbate peroxidase; AA – ascorbic acid; Cys – cysteine; DW – dry weight; FW – fresh weight; Glu – glutamic acid; Gly – glycine; GR – glutathione reductase; GSH – reduced glutathione; GSSG – oxidized glutathione; HPLC – high performance liquid chromatography; LC-MS/MS – liquid chromatography tandem-mass spectrometry; PCs – phytochelatins; PCS – phytochelatin synthase; ROS – reactive oxygen species

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INTRODUCTION

Excess of metals becomes a global problem owing to increasing anthropogenic activities leading to higher toxicity for biota including plants (FARGAŠOVÁ 2012). Among them, cadmium (Cd) is one of the most important contaminants as it accumulates in food chains and has negative impact on cell biochemistry (KOVÁČIK et al. 2015, 2016, 2017a-c). Other common metallic contaminants (limited or no physiological functions in plants) with relatively lower toxicity include nickel (Ni) and lead (Pb) among others (ŠMELKOVÁ et al. 2013; PIOTROWSKA-NICZYPORUK et al. 2015). Metals such as Cu and Zn are essential micronutrients but they are toxic if present in excess both for vascular plants and algae

(TÓTHOVÁ et al. 2011; HAMED et al. 2017).

Excess of metals typically stimulates formation of reactive oxygen species (ROS) which may damage cellular biochemistry if not effectively removed. Plants have developed an array of mechanisms to protect against ROS excess including synthesis of antioxidative molecules such as ascorbic acid (AA) and glutathione (GSH) or metal chelators such as phytochelatins and organic acids (SIMMONS et al. 2009; BRAÜTIGAM et al. 2011; DRESLER et al., 2014). Quantitative accumulation of these metabolites may differ between vascular and non-vascular plants (KOVÁČIK et al. 2017c) and certainly differs among algal species. These metabolites were not frequently reported in the literature owing to low absolute amount in algae requiring precise detection. On the other hand, assay of antioxidative enzymes including ascorbate peroxidase and glutathione reductase is more common owing to inexpensive detection by spectrophotometry.

AA and GSH are not only efficient antioxidants for ROS removal but GSH also serves as a substrate for the synthesis of phytochelatins (PCs, SIMMONS et al. 2009). Recent research indicates that cross-talk between AA and GSH is more complex in algae (LIN et al. 2016). Manipulation of AA biosynthesis in alga *Chlamydomonas reinhardtii* also revealed that algae possess an efficient system for a manifold increase in ascorbate content under stress conditions which is distinct from land plants (VIDAL-MEIRELES et al. 2017). This is fully in agreement with the assumption that microalgae may not rely on tissue structure present in vascular plants and thus regulation of metabolism must be faster and more efficient.

Despite the wide use of algae for biosorption of metals, their metabolic responses to metallic stress have only rarely been reported. Quantitative changes of common antioxidants including ascorbic acid, GSH and related enzymes (ascorbate peroxidase and glutathione reductase) as well as metal chelators phytochelatins (PCs) and organic acids in selected algal species and their evolutionary comparison with macroalgae, mosses and vascular plants are briefly discussed.

ROLE OF NON-ENZYMATIC ANTIOXIDANTS

ASCORBIC ACID. Typical AA amount in Bryophytes and algae is ca. 0.1 – 0.6 μmol g^{-1} FW but mostly >5 μmol g^{-1} FW (2 – 20 μmol g^{-1} FW) in the leaves of higher/vascular plants (GEST et al. 2013). It should be noted that water content of mosses (ca. 75%) is lower if compared with algae or vascular species (ca. 90 – 95%, J. Kováčik, personal observation) then comparison of the data expressed per g DW and g FW must be done with caution (and g DW is certainly better formulation in this case). This fact is mainly visible if chamomile (1.751 μmol g^{-1} DW) and *S. quadricauda* (0.185 μmol g^{-1} DW, Table 1) control AA values are compared: we see almost 10-fold difference indicating evolutionary differences. On the other hand, even chamomile value (using water content ca. 90% in this species) would be ~0.1751 μmol AA g^{-1} FW which falls within above-mentioned range for algae (0.1 – 0.6 μmol AA g^{-1} FW) and it is clear that this range is not universal. Basal AA content, at least in some algal species, must be lower as also shown by our earlier data if *Coccomyxa* and *Scenedesmus* (assayed by LC-MS/MS) are compared (Table 1). Spectrophotometric determination of AA (see control of *S. acutiformis*, Table 1) is also within mentioned range 0.1 – 0.6 μmol g^{-1} FW. Data from other algal species such as often studied *Chlorella vulgaris* show that AA content may differ in relation to culture and/or analytical detection: values 0.11 – 0.39 mg AA g^{-1} DW (= 0.63 – 2.21 μmol AA g^{-1} DW, YUSOF et al. 2011) and ~500 μg AA g^{-1} DW (= 2.83 μmol AA g^{-1} DW, GOIRIS et al. 2015) were reported. In terms of evolutionary similarities, moss *Taxiphyllum* (control) contained similar amount of AA as found in *S. quadricauda* while chamomile and mainly *Ceratophyllum* values are far higher and confirms differences between non-vascular and vascular species (Table 1). Another strong indication of such variation is visible between *Taxiphyllum* (moss) and *Ceratophyllum* (vascular

plant, Table 1) and this difference was confirmed by two methods (spectrophotometry and HPLC). Surprisingly macroalga *Ulva* contains higher amount of AA than microalgal species and even chamomile leaves (Table 1), urging for further research in this algal group.

Metal excess certainly affects main antioxidants due to altered ROS formation as recently confirmed in *S. quadricauda* exposed to 100 μM Cd over 1 h where increase in AA and ROS generation was observed: application of AA biosynthetic inhibitor (lycorine) depleted Cd-induced AA accumulation and enhanced ROS formation, indicating that AA prevents the appearance of ROS under Cd excess (KOVÁČIK et al. 2017b). Unfortunately algae are mainly studied in terms of their eventual usefulness for biofuel production and responses of AA to metallic stress have only relatively rarely been studied. Few available studies reported an increase in AA under Zn excess in *C. sorokiniana* or *S. acuminatus* after prolonged (7 days) exposure (HAMED et al. 2017) or in *S. acutiformis* after 30 days of exposure to Cd (KOVÁČIK et al. 2017a), reinforcing the assumption that AA synthesis is one of the protective mechanisms under metal excess. Additionally, short-term exposure (24 h) to relatively low Cd and Ni doses (1 or 10 μM) evoked increase in AA not only at 1 μM Cd but also at 10 μM Ni more intensively in young than in old culture (KOVÁČIK et al. 2016). Vascular plants exposed (mainly) to Cd revealed increase in AA or unaltered content under higher metal doses (Table 1). On the other hand, redox-active metals such as Cu evoked rapid depletion of AA in macroalga *Ulva compressa* despite elevated activities of main AA biosynthetic enzymes (see MELLADO et al. 2012 for details). Protective effect of AA in algae has only rarely been studied through exogenous application of AA and it was shown that it may affect Cd uptake but maximum depletion (by 15.6%) required 150 μM AA against 25 μM Cd (EL-NAGGAR & EL-SHEEKH 1998). Indirect manipulation of AA level was also observed in microalgae, e.g. nitric oxide donor (sodium nitroprusside) showed a correlation between NO appearance and AA content in combination with Cd excess in *Coccomyxa subellipsoidea* (KOVÁČIK et al. 2015). Manipulation of AA biosynthesis has only recently been published in algae and *Chlamydomonas reinhardtii* with altered *VTC2* gene (encoding GDP-L-galactose phosphorylase) had AA content lower by 90% and was more susceptible to stress including rapid induction of *VTC2* gene by ROS (VIDAL-MEIRELES et al. 2017). It appears to be evident that algae possess an efficient system for a manifold increase in ascorbate content under stress conditions distinct from land plants (VIDAL-MEIRELES et al. 2017) and further studies could highlight this aspect also under metal excess.

GLUTATHIONE (exactly its reduced form GSH) is an essential component of the ROS removal through AA-GSH cycle and a precursor for phytochelatins (see below). GSH is therefore usually more abundant than AA as confirmed also by our data (cf. control values of *Coccomyxa*, *S. quadricauda* or chamomile leaves in Tables 1 and 2). Evolutionary comparison between algae and mosses or vascular plants indicates lower differences than those for AA (less than 5-fold considering water content ~95%; see controls in Table 2).

In terms of metal excess, comparison of Cd and Ni in *S. quadricauda* (1 or 10 μM over 24 h) revealed depletion in Cd treatments and slower response in Ni treatments (KOVÁČIK et al. 2016) but 1 h of 100 μM Cd had no impact on GSH. Longer maintenance of algae *ex vitro* (under non-sterile conditions) affected response to Cd (see KOVÁČIK et al. 2017b for details). In agreement, low Cd doses (0.12 – 1.9 μM) had no impact on GSH after 6 h of exposure in *Pseudokirchneriella subcapitata* (MACHADO & SOARES 2016) but more extensive GSH changes were observed during short-term (0.5 – 14 h) in *Desmodesmus armatus* under 93 μM Cd (POKORA et al. 2014). On the contrary, in vascular plant, moss or macroalga, Cd and Cu evoked rather elevation of GSH amount (Table 2) which correlated with enhancement of GSH-related biosynthetic enzymes in Cu-treated macroalga *Ulva* (MELLADO et al. 2012). In microalgae, even high Zn doses (600 or 1000 μM) had relatively negligible impact on GSH increase (HAMED et al. 2017). *Coccomyxa subellipsoidea* revealed both decrease and

increase in relation to applied Cd concentration and co-application of nitric oxide donor indicated that GSH and Cd accumulation could be correlated (KOVÁČIK et al. 2015). Dinoflagellate *Lingulodinium polyedrum* exposed to 18 μM Cd showed time-independent (24 and 48 h) depletion (ROMANO et al. 2017) and GSH also decreased in Cd-exposed (24 h) *Chlamydomonas* (BRAÜTIGAM et al. 2011). In *Acutodesmus* (formerly *Scenedesmus*) *obliquus*, Pb doses over 10 μM depleted GSH in all time points investigated (PIOTROWSKA-NICZYPORUK et al. 2015) though absolute GSH amount (per g of biomass) was not quantified and cannot be compared with other species. Impact of metals on GSH accumulation depends on the exposure time and applied metal/concentration in various microalgae and the action of Cd seems to be more prominent than that of Ni or Zn (Table 2). Of course, GSH quantitative changes are affected not only by its synthesis but also by consumption for phytochelatin synthesis and/or GSSG-GSH enzymatic conversion through glutathione reductase as mentioned below.

PHYTOCHELATINS (PCs) are oligopeptides synthesized from GSH by plants including algae (hence the prefix phyto-) which are able to bind various metals. They usually contain amino acids $(\gamma\text{-Glu-Cys})_n$-Gly where n = 2-11 (for their synthesis and identification see e.g. PERALES-VELA et al. 2006; SIMMONS et al. 2009). Microalgae typically contains short PCs such as PC2 and PC3 but longer chains (PC4) or their derivatives (CysPCs) were detected in *Chlamydomonas* (BRAÜTIGAM et al. 2011), dinoflagellate *Lingulodinium polyedrum* (ROMANO et al. 2017) or macroalga *Ulva* (MELLADO et al. 2012). In terms of basal (control) values, their accumulation in unstressed algae is lower than that of GSH, often even 100- or 1000-fold differences were observed (e.g. *Coccomyxa* and *Scenedesmus* as detected by LC-MS/MS, cf. Tables 2 and 3). Surprisingly, macroalga *Ulva* contained almost identical amount of PC2 and GSH in control thalli (considering water content ~90%, cf. Tables 2 and 3) while chamomile, a vascular plant, shows amount of PCs (PC2+PC3) ca. 15-fold lower in comparison with GSH (cf. Tables 2 and 3).

Accumulation of PCs is mainly enhanced by excess of Cd, including the activation of enzyme phytochelatin synthase (PCS, SIMMONS et al. 2009). In agreement, microalga *Coccomyxa subellipsoidea* revealed almost 150-fold increase in PC2 in response to 10 and 100 μM Cd over 24 h while GSH amount rather decreased (KOVÁČIK et al. 2015). In *S. quadricauda*, 1 and 10 μM Cd treatment over 24 h evoked over 25-fold elevation of PC2 and GSH also decreased Cd concentration-dependently (KOVÁČIK et al. 2016). At the same time, (though it was argued that Ni is the 6th most efficient PCS inducer), Ni had no impact on PC2 accumulation in *S. quadricauda* (KOVÁČIK et al. 2016). Elevation of PC2 was even detected after 1 h of 100 μM Cd excess in *S. quadricauda* (KOVÁČIK et al. 2017b). We did not observe PC3 or longer chains in *Scenedesmus* but *Chlamydomonas reinhardtii* exposed to 70 μM Cd up to 48 h produced not only PC3 and PC4 but also Cys(PCs) derivatives which increased more pronouncedly in responses to Cd than respective PCs (SIMMONS et al. 2009). *Acutodesmus armatus* produced PC2 and PC3 mainly but also small amount of PC4 in response to Cd was detected (POKORA et al. 2014). Also dinoflagellate *Lingulodinium polyedrum* produced PC3 and PC4 strongly during short-term (24 h) exposure to relatively low Cd doses 9 – 27 μM (ROMANO et al. 2017) and marine macroalga *Ulva* produced rather PC2 than PC3 or PC4 in response to Cu (MELLADO et al. 2012). In vascular plant chamomile, the same method as used for *Scenedesmus* (LC-MS/MS, KOVÁČIK et al. 2016) showed strong induction of PC2 and PC3 in response to Cd (over 10-fold) with higher absolute values of PC3 (see Table 3 and KOVÁČIK et al. 2014). As in the case of GSH mentioned above, also PC2 changes in *Coccomyxa subellipsoidea* were affected by Cd and co-application of nitric oxide donor, indicating that accumulation of Cd and thiols could be correlated (KOVÁČIK et al. 2015). It is clear that some algal species produced PCs similar to those in vascular plants (PC2-3) while inter-specific differences among algae and production of CysPCs derivatives indicate evolutionary differences requiring further research.

Tab. 1: Metal-induced quantitative changes of ascorbic acid (AA) in selected microalgae (macroalga [x], moss [xx] and vascular plant [xxx] for comparison). Values shown as graphs in the cited papers were deducted as exactly as possible.

Species	Applied metal (µM)	Duration	AA content (µmol g^{-1} FW or DW) (control value in brackets)	Reference
Coccomyxa subellipsoidea	10 and 100 Cd	24 h	0.0058 and 0.0026 FW (0.006 FW)	Kováčik et al. 2015
Scenedesmus quadricauda	1 and 10 Cd	24 h	0.817 and 3.139 DW (0.185 DW)*	Kováčik et al. 2016
Scenedesmus quadricauda	1 and 10 Ni	24 h	0.192 and 1.391 DW (0.185 DW)*	Kováčik et al. 2016
Scenedesmus acutiformis	1 and 10 Cd	30 days	0.132 and 0.178 FW (0.117 FW)	Kováčik et al. 2017a
Scenedesmus acuminatus	600 or 1000 Zn**	7 days	0.406 FW (0.247 FW)	Hamed et al. 2017
Chlorella sorokiniana	600 or 1000 Zn**	7 days	0.441 FW (0.229 FW)	Hamed et al. 2017
Scenedesmus quadricauda	100 Cd	1 h	0.597 (0.242 DW)	Kováčik et al. 2017b
Ulva compressa [x]	10 Cu	1 – 7 days	<0.5 DW (4.5 DW)	Mellado et al. 2012
Taxiphyllum barbieri [xx]	10 and 100 Cd	24 h	0.249 and 0.254 DW (0.163 DW)	Kováčik et al. 2017c
Ceratophyllum demersum [xxx]	10 and 100 Cd	24 h	18.23 and 23.66 DW (15.16 DW)	Kováčik et al. 2017c
chamomile leaves [xxx]	60 Cd	48 h	1.582 DW (1.751 DW)	Kováčik et al. 2014

* marked as "young" culture in the cited paper, ** applied dose unclear from the cited paper

Tab. 2: Metal-induced quantitative changes of reduced glutathione (GSH) in selected microalgae (macroalga [x], moss [xx] and vascular plant [xxx] for comparison). Values shown as graphs in the cited papers were deducted as exactly as possible.

Species	Applied metal (µM)	Duration	GSH content (µmol g^{-1} FW or DW) (control value in brackets)	Reference
Coccomyxa subellipsoidea	10 and 100 Cd	24 h	0.091 and 0.036 (0.053 FW)	Kováčik et al. 2015
Scenedesmus quadricauda	1 and 10 Cd	24 h	0.618 and 0.348 DW (0.934 DW)*	Kováčik et al. 2016
Scenedesmus quadricauda	1 and 10 Ni	24 h	0.514 and 0.859 DW (0.934 DW)*	Kováčik et al. 2016
Scenedesmus acuminatus	600 or 1000 Zn**	7 days	0.10 FW (0.05 FW)	Hamed et al. 2017
Chlorella sorokiniana	600 or 1000 Zn**	7 days	0.14 FW (0.09 FW)	Hamed et al. 2017
Lingulodinium polyedrum	18 Cd	24 and 48 h	0.5 DW (1.5 DW)	Romano et al. 2017
Chlamydomonas reinhardtii	70 Cd	24 h	0.05 FW (0.1 FW)	Braütigam et al. 2011
Scenedesmus quadricauda	100 Cd	1 h	0.620 (0.569 DW)	Kováčik et al. 2017b
Ulva compressa [x]	10 Cu	1 – 7 days	1.5 – 6.0 DW (1.5 DW)	Mellado et al. 2012
Physcomitrella patens [xx]	10 Cd	3 days	0.88 FW (0.3 FW)	Hermsen et al. 2010
chamomile leaves [xxx]	60 Cd	48 h	21.7 DW (3.79 DW)	Kováčik et al. 2014

* marked as "young" culture in the cited paper, ** applied dose unclear from the cited paper

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Tab. 3: Metal-induced quantitative changes of phytochelatins of various chain length in selected microalgae (macroalga [x] and vascular plant [xx] for comparison). Values shown as graphs in the cited papers were deducted as exactly as possible.

Species	Applied metal (µM)	Duration	PC content (nmol g^{-1} FW or DW) (control value in brackets)	Reference
phytochelatin 2				
Coccomyxa subellipsoidea	10 and 100 Cd	24 h	2.171 and 4.378 FW (0.029 FW)	Kováčik et al. 2015
Scenedesmus quadricauda	1 and 10 Cd	24 h	163.52 and 213.46 DW (8.047 DW)*	Kováčik et al. 2016
Scenedesmus quadricauda	1 and 10 Ni	24 h	6.863 and 7.214 DW (8.047 DW)*	Kováčik et al. 2016
Chlamydomonas reinhardtii	70 Cd	24 h	19.04 FW (33.3 FW)	Braütigam et al. 2011
Scenedesmus quadricauda	100 Cd	1 h	18.49 (6.165 DW)	Kováčik et al. 2017b
Ulva compressa [x]	10 Cu	1 – 7 days	250 – 600 FW (100 FW)	Mellado et al. 2012
chamomile l eaves [xx]	60 Cd	48 h	794 DW (53.8 DW)	Kováčik et al. 2014
phytochelatin 3				
Chlamydomonas reinhardtii	70 Cd	24 h	19.04 FW (9.52 FW)	Braütigam et al. 2011
Lingulodinium polyedrum	9 – 27 Cd	24 h	64.9 – 31.3 DW (3.1 DW)	Romano et al. 2017
Ulva compressa [x]	10 Cu	1 – 7 days	5 – 35 FW (5 FW)	Mellado et al. 2012
chamomile leaves [xx]	60 Cd	48 h	2435 DW (206 DW)	Kováčik et al. 2014
phytochelatin 4				
Lingulodinium polyedrum	9 – 27 Cd	24 h	118.7 – 89.1 DW (1.2 DW)	Romano et al. 2017
Ulva compressa [x]	10 Cu	1 – 7 days	35 – 53 FW (30 FW)	Mellado et al. 2012

* marked as "young" culture in the cited paper

INVOLVEMENT OF ENZYMATIC ANTIOXIDANTS

Plant cell possesses an array of enzymes to scavenge ROS formations due to metal excess (and other stresses) which were reviewed many times before. Here I will mention mainly ascorbate peroxidase (APX) as the main AA-decomposing enzyme and glutathione reductase (GR) regenerating GSH from its oxidized state (GSSG). Both these enzymes are essential for hydrogen peroxide removal through ascorbate-glutathione cycle (see e.g. GEST et al. 2013 for details).

Comparison of APX activity in selected microalgal species revealed roughly similar control values (at the level of hundreds of nmol min^{-1} mg^{-1} protein, see Table 4) and lower value in comparative vascular plant chamomile (assayed by identical method as algae). Also *A. obliquus* data are lower compared to other microalgae presented in Table 4. Exceptionally high basal APX activity in *Coccomyxa subellipsoidea* could account for low AA content in this species (cf. Table 1 and 4) and such high APX activity (3.76 U mg^{-1} protein = 3760 nmol min^{-1} mg^{-1} protein) has also been reported in other *Coccomyxa* species (RUIZ-DOMÍNGUEZ et al. 2015). Depletion of APX activity after very short (1 h, KOVÁČIK et al. 2017b) or short (24 h) impact of Cd (KOVÁČIK et al. 2015) was reported in microalgae or water moss *Taxiphyllum* (KOVÁČIK et al. 2017c). Longer studies with microalgae showed either elevation (7 days, HAMED et al. 2017) or unaltered activity (30 days, KOVÁČIK et al.

2017a) in response to various metals. One of few combined studies revealed interesting responses in microalga *C. subellipsoidea* where combination of Cd with nitric oxide donor elevated AA content and APX activity (see KOVÁČIK et al. 2015 for details), indicating direct relation between these parameters. In agreement, APX not only use AA as a cofactor for hydrogen peroxide removal but is also inactivated by low AA content (GEST et al. 2013, see also evolution and significance of APX in this work). At the gene expression level, APX expression was variously affected by metals in microalga *C. reinhardtii* (after 2 weeks of exposure): Cd doses 10 – 40 μM evoked dose-dependent increase while Cu or Hg doses over 3 or 5 μM had strongly negative impact (NOWICKA et al. 2016), indicating metal-specific action even in the given species.

Glutathione reductase (GR) is important e.g. for providing reduced glutathione (GSH from GSSG) for regeneration of oxidized ascorbate generated through ascorbate-glutathione cycle (see e.g. GEST et al. 2013 for details). Basal activities of this enzyme appear to be more variable among various algal species than APX mentioned above. I mainly note that activity at the level of 0.5 nmol min^{-1} mg^{-1} protein (PIOTROWSKA-NICZYPORUK et al. 2015) is far lower than in other species from the (former) genus *Scenedesmus* or others, arising the question about quantitative assay: however, low impact of Pb on GR activity in the given species indicates tolerance of *Acutodesmus* to even 100 μM Pb (Table 4). Unaltered or elevated activity of this enzyme was also reported in various plants and microalgae and only short-term (1 h) exposure to Cd evoked depletion (KOVÁČIK et al. 2017b). The complexity of interaction between AA and GSH is further visible in a recent study using *Chlamydomonas reinhardtii* where overexpression of dehydroascorbate reductase (converting oxidized to reduced AA at the expense of GSH) increased GSH/GSSG ratio, glutathione pool and APX or GR activities and protected algae against photoxidative stress (LIN et al. 2016). Algae with increased antioxidative protection could provide more tolerant strains for metal absorption in the future.

Tab. 4: Metal-induced quantitative changes of AA- and GSH-related enzyme activities (nmol min^{-1} mg^{-1} protein) in selected microalgae (moss [x] and vascular plant [xx] for comparison). Values shown as graphs in the cited papers were deducted as exactly as possible.

Species	Applied metal (μM)	Duration	Enzyme activity (control value in brackets)	Reference
ascorbate peroxidase				
Coccomyxa subellipsoidea	10 and 100 Cd	24 h	615 and 459 (1291)	Kováčik et al. 2015
Scenedesmus acuminatus	600 or 1000 Zn*	7 days	266 (133)	Hamed et al. 2017
Chlorella sorokiniana	600 or 1000 Zn*	7 days	720 (400)	Hamed et al. 2017
Acutodesmus obliquus	1 – 10 – 100 Pb	24 h	24 – 21.5 – 18.9 (24)	Piotrowska-Niczyporuk et al. 2015
Scenedesmus acutiformis	1 and 10 Cd	30 days	192 and 205 (204)	Kováčik et al. 2017a
Scenedesmus quadricauda	100 Cd	1 h	287 (412)	Kováčik et al. 2017b
Taxiphyllum barbieri [x]	10 an 100 Cd	24 h	196 and 185 (256)	Kováčik et al. 2017c
chamomile leaves [xx]	60 Cd	48 h	51.4 (53)	Kováčik et al. 2014
glutathione reductase				
Coccomyxa subellipsoidea	10 and 100 Cd	24 h	173 and 119 (141)	Kováčik et al. 2015
Scenedesmus acuminatus	600 or 1000 Zn*	7 days	62 (43)	Hamed et al. 2017
Chlorella sorokiniana	600 or 1000 Zn*	7 days	155 (80)	Hamed et al. 2017
Acutodesmus obliquus	1 – 10 – 100 Pb	24 h	0.55 – 0.48 – 0.45 (0.51)	Piotrowska-Niczyporuk et al. 2015
Scenedesmus acutiformis	1 and 10 Cd	30 days	292 and 375 (304)	Kováčik et al. 2017a
Scenedesmus quadricauda	100 Cd	1 h	322 (536)	Kováčik et al. 2017b
chamomile leaves [xx]	60 Cd	48 h	25.8 (27.5)	Kováčik et al. 2014

* applied dose unclear from the cited paper

ORGANIC ACIDS

Aliphatic organic acids are potential chelators of metals in various plants (DRESLER et al. 2014). Also water moss *Taxiphyllum* and vascular aquatic plant *Ceratophyllum* revealed elevated production of citrate and malate in response to Cd with molar ratio 1.2:1 and 4:1, indicating potential involvement in Cd chelation (KOVÁČIK et al. 2017c). In microalga *C. subellipsoidea*, it was observed that quantitative changes of malate evoked by Cd and co-application of nitric oxide donor showed potential correlation with Cd accumulation (KOVÁČIK et al. 2015). Comparison of *Scenedesmus* cultures of various age exposed to Cd or Ni revealed that organic acids including citrate and malate decreased or increased in relation to age of the culture and the impact of Ni was more pronounced compared to Cd (KOVÁČIK et al. 2016). Also short-term (1 h) of Cd presence affected often considerably accumulation of Krebs cycle acids (KOVÁČIK et al. 2017b). Accumulation of organic acids in microalgae has only rarely been studied probably owing to low quantitative levels (up to ten of $\mu g\ g^{-1}$ FW in comparison with vascular plants) requiring sensitive detection techniques. Further studies focused on inter-specific comparison among microalgae and macroalgae could certainly reveal the impact of metals on organic acids and their eventual role in metal chelation.

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